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# Vaccination of Cats for Feline Rhinotracheitis Results in a Quantitative Reduction of Virulent Feline Herpesvirus-1 Latency Load after Challenge

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We recently described the construction of a feline herpesvirus-1 (FHV-1) recombinant vaccine strain, FHV$\beta$-galgIgE$\Delta$, containing a deletion in both the gI and the gE genes. We also reported comparative tests of its safety and efficacy in cats. These cats were unvaccinated or subcutaneously vaccinated with FHV$\beta$-galgIgE$\Delta$, its isotypic parent strain (FHV-1SA), or a commercial FHV-1 vaccine strain (FHV-1CV) and subsequently challenged with a virulent field strain of FHV-1. Here we report the determination of FHV-1 field virus latency load after challenge in the same experimental animals. FHV-1 specific quantitative PCR was carried out on feline genomic DNA isolated from trigeminal ganglia, olfactory bulbs, and brain stems. We have determined that a reduction in field virus latency load in cats vaccinated subcutaneously with wild-type FHV-1 strains prior to challenge is dependent upon glycoproteins gI and gE deleted in FHV$\beta$-galgIgE$\Delta$. 

Sites of primary alphaherpesvirus infection include the ocular, oronasal, and urogenital epithelia, depending upon the virus species and its tropism. As the acute infection progresses, virus is transported across synapses into neurons that innervate the epithelial mucosae (*1*). Latent infection of the nervous system after the acute phase of infection is common to all alphaherpesviruses (*2*). Furthermore, latent HSV-1,2, VZV, PRV, BHV-1, EHV-1, and feline herpesvirus-1 (FHV-1) have been characteristically isolated from the trigeminal ganglia of their respective host species (*1, 3–7*).

Feline herpesvirus-1, an alphaherpesvirus, is a significant pathogen limited to the family *Felidae.* FHV-1 primarily affects the upper respiratory system of cats. Ocular disease, abortion in pregnant queens, neurological disorders, and virus generalization, with mortality reaching 50% in kittens, have also been observed. Lifelong latent infection follows acute respiratory disease, and reactivated latent FHV-1 plays an important role in virus perpetuation and spread (*8*). Frequently, during periods of stress such as change of housing, queening, or lactation, latent virus can reactivate. Although clinical signs may be absent or mild, recrudescent animals can shed infectious virus for up to 1 week (*9*).

Previously we described the construction of FHV$\beta$-galgIgE$\Delta$ and showed that this strain was efficacious when administered by the subcutaneous or oronasal route (*10, 11*). While FHV$\beta$-galgIgE$\Delta$ administered via the subcutaneous route effectively reduced clinical signs due to virulent challenge, vaccine administration via the oronasal route provided significantly better protection. We also showed that FHV-1CV, a representative commercially available subcutaneously administered vaccine strain, was effective by the subcutaneous route, but highly virulent when administered oronasally (*11*). It has been suggested that such FHV-1 vaccines are not virulent when administered subcutaneously because they do not replicate as efficiently at the cat's normal core body temperature as at cooler sites in the upper respiratory tract (*12*).

As part of this study we have quantitated latent wild-type FHV-1 in the trigeminal ganglia, olfactory bulbs, and brain stem of cats either unvaccinated or subcutaneously vaccinated with FHV-1CV, FHV$\beta$-galgIgE$\Delta$, or FHV-1SA prior to challenge with a virulent field strain of FHV-1. Twenty-one and seven days prior to oronasal challenge with $10^6$ $TCID_{50}$ of FVR-SGE, a virulent challenge strain provided by the USDA, three randomly chosen groups of five specific pathogen-free cats were each inoculated subcutaneously with $10^7$ $TCID_{50}$ of FHV-1CV, FHV-1SA, or FHV$\beta$-galgIgE$\Delta$. Virus neutralization (VN) titers following subcutaneous vaccination as previously described were moderate with no significant differences between VN titers induced by any of the inoculated strains (*11*). Post-challenge clinical scores and virus shedding were substantially reduced in immunized cats compared to control cats. Cats receiving FHV$\beta$-galgIgE$\Delta$ had a higher fre-

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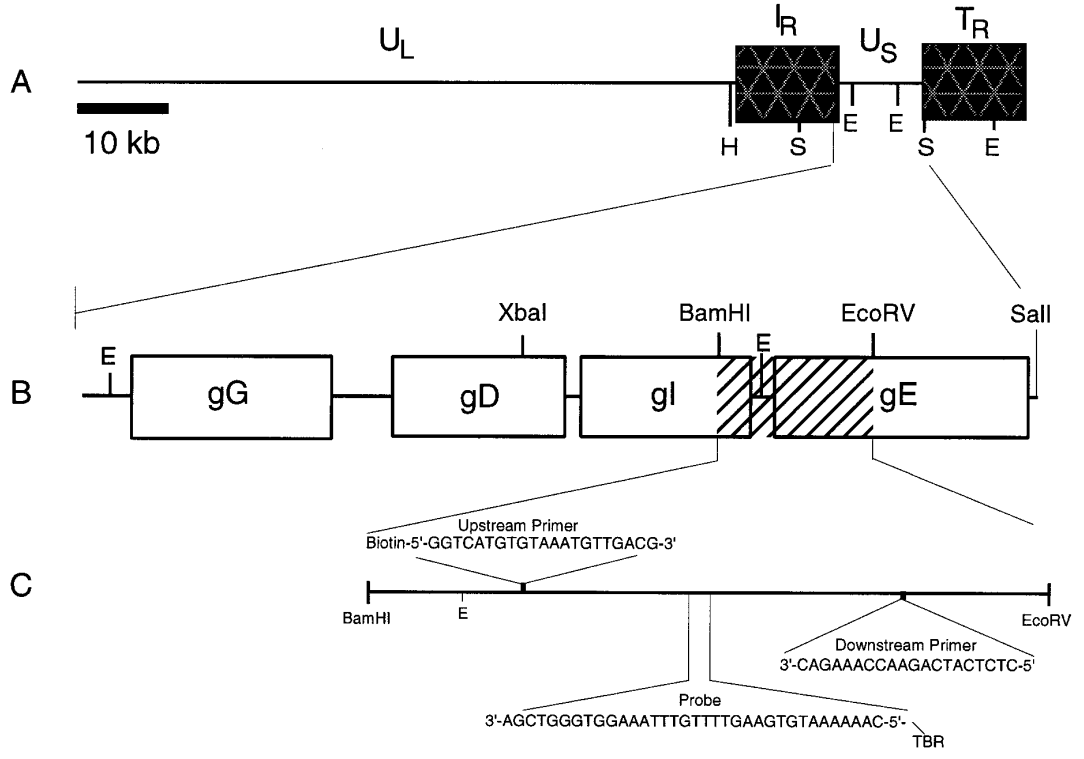


FIG. 1. Locations of specific primer and probes used for wild-type FHV-1 QPCR assay. (A) Diagram of the FHV-1 genome. $U_L$, unique long region; $I_R$, internal repeat (hatched box); $U_S$, unique short region; $T_R$, terminal repeat (hatched box). E, *Eco*RI restriction site; S, *Sal*I restriction site; and H, *Hin*dIII restriction site. (B) Expanded view of unique short region. Glycoprotein genes are depicted as boxes. Hatched area represents the region deleted in FHV$\beta$-galgIgE$\Delta$. (C) Location of upstream biotinylated primer, downstream primer, and TBR-labeled probe used for the wild-type FHV-1 QPCR assay. Sequences above the line are sense-strand coding. Those below the line are antisense-strand coding.

quency of virus shedding than FHV-1SA or FHV-1CV vaccinees (*11*). Methods used for the care of cats prior to vaccination, for vaccination with FHV-1CV and FHV$\beta$-galgIgE$\Delta$, and for challenge with a field virus strain have previously been described (*10*). Eight weeks after oronasal challenge with virulent FHV-1 (FVR-SGE), cats were anesthetized by subcutaneous inoculation of ketamine hydrochloride and acepromazine maleate and then humanely euthanized with an overdose of pentabarbital sodium administered by intracardiac injection. Prior to euthanasia virus shedding was assessed in all cats. No evidence of viral reactivation was found. Trigeminal ganglia were harvested by sharp excision of tissue located in the cavum trigeminale of the dura mater lateral to the cavernous sinus at the apex of the petrous temporal bone. After collection, these tissues were immediately frozen in liquid nitrogen, placed in cryogenic storage vials, and stored at −80°. Tissues were divided in half prior to DNA isolation. Total DNA from each of the four pieces was isolated by proteinase K and detergent disruption of tissue followed by silica gel spin-column purification as described by the manufacturer (Qiamp kit; Qiagen, Inc.). Total genomic DNA was also isolated from olfactory bulbs and brain stem tissue. Although other DNA isolation methods were attempted, the silica gel column method (Qiamp; Qiagen, Inc.) was used because it did not introduce any potential PCR inhibitors to isolated nucleic acids (data not shown) (*13*).

We previously described a PCR assay for the FHV-1 gE gene that detects as few as 400 genomes of a FHV-1 $gE^+$ strain in 0.1 $\mu$g of total cellular DNA with ethidium bromide staining of a 478-bp PCR product electrophoresed on an agarose gel (*10*). The locations and sequences of primers used are depicted in Fig. 1. This PCR amplification was adapted for electrochemiluminescent quantitation of latent FHV-1 in feline tissue samples. For the quantitative assay, a 478-bp fragment of the FHV-1 gE gene was amplified using an upstream biotinylated primer (Fig. 1). Biotinylation enabled the capture of specific PCR products on streptavidin-coated magnetic beads. A 35-bp DNA probe (5′-CAAAAAATGTGAAGTTTT-GTTTAAAGGTGGGTCGA-3′) with TBR (tris(2′,2′-bipyridine)-ruthenium(II) chelate) attached to the 5′-end and sequence complementary to the wild-type FHV-1 strains including FVR-SGE, FHV-1CV, and FHV-1SA provided a wild-type specific electrochemiluminescent reporter (Fig. 1). Post-PCR hybridization of the reporter probe to the biotinylated PCR product strand was done for 15 min at 55° after a brief denaturation at 94° for 5 min. Excess streptavidin-coated magnetic beads (Dynal, M450) were added to the hybridization mixture, which was incubated an additional 15 min at 55°. The entire mixture was di-

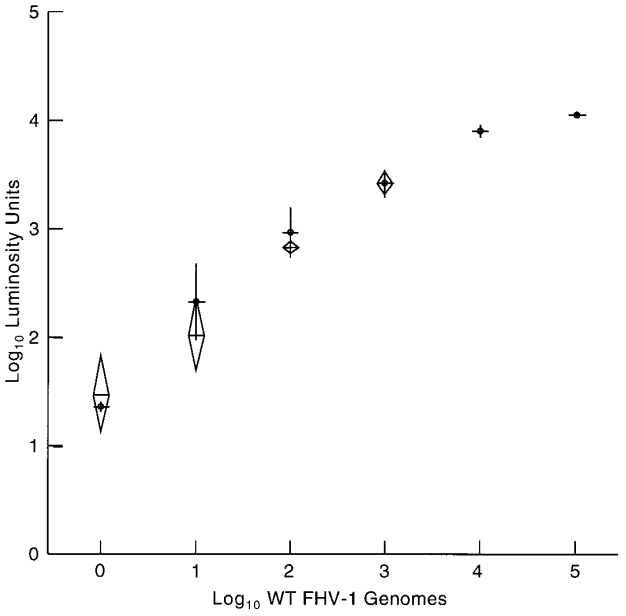


FIG. 2. FHV-1 virion DNA standard curve. Six sets of serial dilutions containing known concentrations of highly purified FHV-1 virion DNA were assayed in the presence or absence of 105 ng of TG DNA using the quantitative PCR assay described in the text. The average luminosity value and standard deviation for each dilution of the three sets assayed in the presence of TG DNA is depicted as ⊕, where standard deviation is indicated by the vertical bar. The average luminosity values and standard deviations for each dilution of three sets of FHV-1 virion DNA assayed in the absence of TG DNA are depicted as ◇, with standard deviation indicated by the vertical apices of the diamond. Electrochemiluminescence was measured in luminosity units. FHV-1 genomic copy number was determined by spectrophotometric and fluorimetric analysis of virion DNA prior to dilution. Luminosity values for "with and without" TG DNA sets were normalized to the manufacturer's standards (CV = 5%) at the $10^3$ genome equivalent value to adjust for instrument drift and recalibration between the two series of assays. FHV-1 virion DNA was isolated as previously described (*10*).

luted to 0.7 ml with buffer containing tripropylamine, an electrochemiluminescent activator. The QPCR assay was carried out in an automated electrochemiluminescence detector (Perkin–Elmer QPCR 5000) (*14*).

Initial sample FHV-1 DNA concentration was determined by interpolation of a standard curve. A standard curve was prepared for each set of tissue samples assayed by amplifying and determining the luminosity associated with known initial FHV-1 genomic DNA concentrations (*15*). Specific detection of FHV-1 DNA was not significantly affected by the presence of cellular DNA. Figure 2 depicts standard curve data from six separate QPCR assays performed in the presence or absence of added feline trigeminal ganglionic (TG) DNA. In one series of experiments, three serial dilution sets each containing 1, 10, $10^2$, $10^3$, $10^4$, and $10^5$ genome equivalents of purified FHV-1 virion DNA were individually prepared, combined with a constant amount of TG DNA (105 ng), and assayed. Three serial dilution series containing 1, 10, $10^2$, and $10^3$ genome equivalents of purified FHV-1 virion DNA were also assayed with no added TG DNA. In addition, known concentrations of FHV-1 genomic DNA comparable to those used to construct the standard curve in Fig. 2 were accurately quantitated in "spiked" solutions containing as much as 5 $\mu$g of TG, olfactory bulb, or brain stem total DNA (data not shown).

In this study, FHV-1 latency load was determined as FHV-1 genome equivalents per microgram of TG DNA. Estimates of the number of latent FHV-1 genomes/100 cells are based on the following analysis. Assuming the haploid feline genome contains on the order of $3.5 \times 10^9$ bp (*16*), and each DNA base pair has a molecular weight of approximately 660 Da (Avogadro's number $= 6.023 \times 10^{23}$ molecules/g molecular weight equivalent), it can be determined that one haploid genome weighs approximately 3.8 pg and 1 $\mu$g of feline genomic DNA will contain approximately 260,000 haploid genome equivalents. In order to calculate the number of FHV-1 genomes per cell, we have also assumed that ganglionic cells are diploid.

Latent FHV-1 was recovered at significantly higher concentrations in trigeminal ganglia from cats that were either not vaccinated or vaccinated subcutaneously with FHV$\beta$-galgIgE$\Delta$ than in ganglia from cats that were vaccinated with either FHV-1SA or FHV-1CV ($P < 0.01$; Table 1; *F*-test) (*17*). Only extremely low levels (1–23 copies/130,000 cells) of FHV-1 DNA were detected in either olfactory bulbs or brain stem tissue.

There is an approximately 10-fold difference between the mean field virus latency load after challenge of cats subcutaneously vaccinated with the parent or commercial FHV-1 strains and that of those subcutaneously vaccinated with FHV$\beta$-galgIgE$\Delta$ (Table 1). Since only the gI and gE genes have been interrupted in FHV$\beta$-galgIgE$\Delta$, these genes or their glycoprotein products must play a role in reducing the field virus latency load after challenge in the trigeminal ganglia of FHV-1CV and FHV-1SA vaccinees.

Previously we showed that FHV-1 vaccine strains lacking glycoproteins gI and gE produce plaques of reduced size compared to those of the wild-type strain FHV-1SA, suggesting that the mutant virus spreads poorly by syncytium formation (*10*). It was also determined that subcutaneously inoculated FHV-1SA was invasive, could spread from the inoculation site to epithelial tissues, and could produce a productive infection that resulted in detectable virus shedding (*10*). Vaccine virus spread from the subcutaneous inoculation site may be required for protection against field virus colonization of the trigeminal ganglion in subcutaneously vaccinated cats after challenge and may explain the inability of FHV$\beta$-galgIgE$\Delta$ to protect against it. In this study the influence of vaccine strain latency (precolonization of the trigeminal ganglia) on protection from field virus latency after challenge in FHV-1CV and FHV-1SA vaccinees could not be established, since latent virus detected in both FHV-1CV and FHV-1SA vaccinees could be either vaccine or challenge virus (*18*). It is also possible that stimulation of a mucosal or

TABLE 1

Latency Load in Tissues of Previously Immunized Cats after Virulent FHV-1 Challenge

| Vaccination group | N | Trigeminal ganglion: Mean latency load (FHV-1 genomes/$\mu$g ganglionic DNA)[a] | Trigeminal ganglion: Latent FHV-1/100 cells[b] | Olfactory bulb: Mean latency load (FHV-1 genomes/$\mu$g olfactory bulb DNA)[a] | Olfactory bulb: Latent FHV-1/100 cells[b] | Brain stem: Mean latency load (FHV-1 genomes/$\mu$g brain stem DNA)[a] | Brain stem: Latent FHV-1/100 cells[b] |
|---|---|---|---|---|---|---|---|
| FHV-1CV | 5 | 926 $\pm$ 1,634 (3,825) | 0.71 | 18.20 $\pm$ 30 (69) | 0.014 | 2.06 $\pm$ 3 (7) | 0.002 |
| FHV$\beta$-galgIgE$\Delta$ | 5 | 7,211 $\pm$ 10,799 (26,303) | 5.5 | 14.40 $\pm$ 15 (36) | 0.011 | 16.40 $\pm$ 14 (34) | 0.013 |
| FHV-1SA | 5 | 565 $\pm$ 576 (1,188) | 0.43 | 10.40 $\pm$ 7 (15) | 0.008 | 1.0 $\pm$ 2 (5) | 0.001 |
| Unvaccinated | 5 | 7,276 $\pm$ 15,843 (35,595) | 5.6 | 23.40 $\pm$ 24 (60) | 0.018 | 4.14 $\pm$ 4 (10) | 0.003 |

[a] Average latency load was determined for all cats by QPCR analysis of feline genomic DNA extracted from nervous tissue and is shown with the standard deviation. The range is in parentheses.

[b] Based on 260,000 haploid genome equivalents/$\mu$g of feline cellular DNA.

cell-mediated immune response at the portal of entry was important in producing the observed protection against increased field virus latency load of FHV-1CV and FHV-1SA vaccinees. If viral spread to the mucosae was a factor in preventing field virus latency, then FHV$\beta$-galgIgE$\Delta$ administered oronasally at the portal of entry would be expected to be more protective.

Reactivation of latent FHV-1 due to stress or the administration of corticosteroids results in renewed viral replication, reinfection of the epithelium, virus shedding, and the potential for FHV-1 infection of other cats (*19*). Reactivation potential may be an important factor in determining vaccine efficacy. At the cellular level, reactivation of HSV-1 appears to be dependent upon a neuronal response to stress such as the administration of corticosteroids or heat shock, but requires the presence of latent HSV-1 within the responding neuron (*20*). Latency-associated transcripts (LATs) of HSV-1 are also found in the majority of herpesvirus-1 neurons of latently infected rabbit and the level of LAT expression appears to be correlated with the number of HSV-1 genomes present (*21*). Furthermore, HSV-1 latency load does not appear to change over time or after reactivation in latently infected rabbits, suggesting that only a small percentage of infected neurons is involved in reactivation (*22*). If reactivation potential was directly dependent upon latency load, then latency load would be a good parameter for determining vaccine efficacy. However, a direct correlation between increased latency load and increased reactivation potential has not yet been proven.

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